

## METHODS OF FACILITATING VASCULAR GROWTH

Margaret A. Schwarz, Fangrong Zhang, and Sarah A. Gebb

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### Related Applications

This application claims priority from United States Provisional Application  
Serial No. 60/108,435, filed November 13, 1998, the disclosure of which is  
10 incorporated by reference herein in its entirety.

### Field of the Invention

The present invention concerns methods of facilitating vascular growth in a  
subject, such as a subject at risk for ischemic reperfusion injury, or a newborn  
15 afflicted with bronchopulmonary dysplasia. Methods of identifying compounds  
useful for the aforesaid treatments are also disclosed.

### Background of the Invention

U.S. Patent No. 5,641,867 to D. Stern et al. (assigned to Columbia University)  
20 describes purified endothelial monocyte activating polypeptide (EMAP) II,  
antibodies that specifically bind to EMAP II, and methods of treating tumors by  
administering EMAP II to an afflicted subject.

U. Knies et al., *Proc. Natl. Acad. Sci. USA* **95**, 12322-12327 (Oct. 1998),  
describes the regulation of endothelial monocyte-activating polypeptide II release by  
25 apoptosis.

### Summary of the Invention

A first aspect of the invention is a method of facilitating vascular growth in a subject, such as in an organ or tissue of the subject, in need of such treatment. The method comprises inhibiting EMAP II activity in the of the subject (*e.g.*, in the aforesaid organ or tissue) by an amount effective to stimulate vascular growth.

A second aspect of the present invention is a pharmaceutical formulation comprising: an active compound selected from the group consisting of compounds that specifically bind to EMAP II, compounds that inhibit the expression of EMAP II, and EMAP II receptor antagonists; and a pharmaceutically acceptable carrier.

A third aspect of the present invention is a method of screening for compounds useful for facilitating vascular growth in a subject in need thereof. The method comprises: contacting a test compound (*e.g.*, a protein or peptide) to a probe molecule, the probe molecule selected from the group consisting of EMAP II and fragments thereof; and then detecting the presence or absence of binding of the test compound to the probe molecule, the presence of binding indicating the compound may be useful for facilitating vascular growth in a subject.

A fourth aspect of the present invention is a method of screening for compounds useful for facilitating vascular growth in a subject, comprising: contacting a test compound (*e.g.*, an oligonucleotide) to probe molecule, the probe molecule selected from the group consisting of DNA encoding EMAP II, RNA encoding EMAP II, and fragments thereof; and then detecting the presence or absence of binding of the test compound to the probe molecule, the presence of binding indicating the compound may be useful for facilitating vascular growth in the subject.

A fifth aspect of the present invention is a method of screening for compounds useful for facilitating vascular growth in a subject, comprising: determining *in vitro* whether a test compound inhibits expression of EMAP II; the inhibition of expression of EMAP II indicating the compound may be useful for facilitating vascular growth in a subject.

The present invention is explained in greater detail in the specification set forth below.

### **Brief Description of the Drawings**

**Figure 1:** Purification of recombinant (r) EMAP II. REMAP II in a PET28a 6X his-tag system was isolated after IPTG induction from E. coli. Lane one on this coomassie blue gel, is the initial expression of rEMAP II. Lane two represents rEMAP II induction with IPTG and purified rEMAP II in lane three.

**Figure 2:** EMAP II inhibits fetal lung vascular development. Fetal lung xenografts transplanted subcutaneously in immunocompromised mice treated intraperitoneally (IP) with rEMAP II, EMAP II antibody, or vehicle (PBS or rabbit serum respectively) were evaluated for vessel formation after 14 days of implantation using the PECAM-1 antibody. There was a marked inhibition of neovascularization in the transplants of mice receiving rEMAP II (2B) compared to vehicle alone (2A). Analysis of vessel formation, assessed by counting the number of vessels per HPF (averaging counts from 10 HPF per lung implant, n=20 implants / group performed on 4 separate occasions) showed a 56% reduction in neovascularization in animals receiving rEMAP II compared to control (2D) ( $p < 0.0001$ ). This is in sharp contrast to those transplants in animals that received EMAP II antibody where there is a dose dependent 50% increase in vessel counts ( $p < 0.0001$ ) (2C) compared to control (2E) (n=10 / group performed on 3 separate occasions). A reduction in PECAM-1 band and Tie-2 band mRNA by RT-PCR in lung xenografts from mice treated with EMAP II and an increase in PECAM-1 and Tie-2 mRNA in animals treated with EMAP II antibody confirmed the immunohistochemical results (2F). RT-PCR results were normalized using the  $\beta$ -actin as an internal control. Negative controls for PCR amplification of the PECAM-1 and Tie-2 transcripts, without RT, demonstrated no specific PCR product in each rxn (data not shown). Bar = 500  $\mu$ m.

**Figure 3:** Excess EMAP II leads to an alteration in lung epithelial morphogenesis. Lung xenografts in mice treated IP with rEMAP II demonstrated marked lung dysplasia, defined by the presence of flattened epithelial cells in the central airway region (arrows in 3D) and poorly formed peripheral airways (3E,F). This is in sharp contrast to those transplants in mice treated with vehicle where there were well defined bronchi, epithelium, (3A) and distal spaces with attenuated epithelium consistent with alveoli (3 B,C). Bar = 500  $\mu$ m in A,B,D,E; Bar = 250  $\mu$ m in C,F.

**Figure 4:** Excess EMAP II significantly alters cellular differentiation in fetal lung development. Fetal lung xenografts undergo cellular differentiation with the

appearance of type II alveolar cells expressing SP-C after 14 days of implantation in immunocompromised mice (4A,B) in the presence of vehicle alone. In contrast, animals receiving excess rEMAP II IP exhibit no SP-C expression throughout the entire xenograft including those areas that histologically appeared as dysplastic peripheral airways (4C,D). In contrast, an excess of type II cells is found in xenografts in animals treated with the blocking EMAP II antibody (4E,F). This is indicative of a marked effect of EMAP II on lung epithelial maturation. These in situ hybridization findings were supported by a reduction in SP-C RNA in lung xenografts from mice treated with EMAP II compared to control and an increase in animals treated with EMAP II antibody (4G). Assessment of type I alveolar cell markers revealed a slight elevation in T1- $\alpha$  in xenografts treated with rEMAP II compared to control. There was also a marked reduction of T1- $\alpha$  in xenografts in animals treated with the blocking EMAP II, the inverse of the high level of in situ hybridization of SP-C in type II cells (4G). RT-PCR results were normalized using the housekeeping gene  $\beta$ -actin (350 bp). Negative controls, demonstrated no band (data not shown). Bar = 500  $\mu$ m in A,C,E; 250  $\mu$ m in B,D,F.

**Figure 5:** Effect of excess rEMAP II on glycogen within the xenografts. Glycogen (denoted as the magenta color of the PAS stain) is markedly elevated in those lung transplants in mice treated IP with rEMAP II (5D-F). In contrast, xenografts in vehicle treated mice contain a normal distribution of glycogen, consistent with that seen in the late saccular stage (5A-C). Thus, presence of excess EMAP II appears to halt lung formation in the glycogen-rich epithelial stage, associated with the beginning of the canalicular (vascular) stage, just prior to alveolar type II epithelial cell differentiation. Bar = 500  $\mu$ m in A,B,D,E; 250  $\mu$ m C,F.

**Figure 6:** In situ hybridization of EMAP II exhibits a peri-epithelial cyst pattern of EMAP II expression (6A) that is confirmed by immunohistochemistry (6B). In contrast to minimal production of EMAP II in the distal mesenchyme, the mesenchymal cells in close contact with the epithelium display a marked increase in EMAP II as indicated by the arrows in 6B. Therefore, it appears that in addition to expression of EMAP II in the epithelial and mesenchymal cells, it is the actual cell-cell interaction between the epithelial and mesenchymal cells that increases EMAP II's expression. Bar = 500  $\mu$ m in A,C; 250  $\mu$ m in B,D.

**Figure 7:** Effect of EMAP II on epithelial cyst formation in co-cultures. Co-cultures of epithelial and mesenchymal cells exposed to excess rEMAP II have a marked 71% decrease of epithelial cyst formation (7B) in a dose-dependent fashion (7D) as compared to vehicle alone (7A). In addition, in contrast to normal cyst formation noted in the inset of figure 7A, where the cyst is enclosed by a flattened cell population (arrows in 7A inset) consistent with the laminin cell population surrounding the cyst, those co-cultures treated with EMAP II (7B, inset) or EMAP II antibody (7C, inset) lacked the normal cyst formation. In contrast, co-cultures grown in the presence of EMAP II antibody reflect a 54% increase in cyst formation (7C) that is also dose-dependent in nature (7E). We speculate that excess EMAP II interferes with epithelial cyst formation and stability. (n=7 / group performed on 4 different occasions) Bar = 500  $\mu$ m in A-C; 250  $\mu$ m insets of A-C.

#### **Detailed Description of Preferred Embodiments**

As noted above, a first aspect of the invention is a method of facilitating vascular growth in a subject in need of such treatment. The method comprises inhibiting EMAP II activity in the subject by an amount effective to stimulate vascular growth.

Vascular growth may be inhibited in any suitable organ or tissue, including but not limited to lung, kidney, heart, aorta, gastrointestinal tract, brain, liver, etc. The inhibition may be specific or general, primarily influenced by the manner of administration as discussed below. Applicants invention is not intended to be limited to any particular theory of vascular growth, and hence this term is intended to be construed generally, encompassing any type of vascular growth such as vasculogenesis, angiogenesis, etc.

Subjects that may be treated by the present invention include any subject, human or adult, for which it is desired to facilitate vascular growth. Such subjects include subjects at risk for ischemic reperfusion injury to an organ such as those described above (*e.g.*, in the case of transplant, low blood pressure, cardiac arrest, etc.), newborn subjects afflicted with bronchopulmonary displaysia, subjects afflicted with pulmonary hypertension, subjects afflicted with lung hypoplasia, etc.

While subjects treated by the present invention are primarily human subjects, the invention may also be carried out on other animal subjects such as dogs, cats, horses, etc. for veterinary purposes.

The inhibiting step may be carried out by any suitable means. For example, it may be carried out by administering a compound that specifically binds to EMAP II to the subject in an amount effective to stimulate vascular growth. Such compounds may be antibodies (including polyclonal and monoclonal antibodies, antibody fragments, humanized or chimeric antibodies, etc. that retain the combining region that specifically binds to EMAP II). The antibodies may be of any type of immunoglobulin, including but not limited to IgG and IgM immunoglobulins. The antibodies may be of any suitable origin, such as chicken, goat, rabbit, horse, etc., but are preferably mammalian and most preferably human. The antibody may be administered directly or through an intermediate that expresses the antibody in the subject. Examples of EMAP II antibodies are provided in U.S. Patent No. 5,641,867 to Stern et al., the disclosure of which is incorporated herein by reference. Examples of the different forms of therapeutic antibodies are given in U.S. Patent No. 5,622,700, the disclosure of which is incorporated herein by reference.

The inhibiting step may be carried out by downregulating EMAP II expression in the subject by an amount effective to stimulate vascular growth in the lungs of the subject. Compounds useful for downregulating EMAP II expression are, in general, antisense oligonucleotides that bind to EMAP II mRNA and disrupt translation thereof, or oligonucleotides that bind to EMAP II DNA and disrupt transcription thereof. Such oligonucleotides may be natural or synthetic (such as described in U.S. Patent No. 5,665,593 to Kole, the disclosure of which is incorporated by reference herein in its entirety), and are typically at least 4, 6 or 8 nucleotides in length, up to the full length of the corresponding DNA or mRNA. Such oligonucleotides are selected to bind to the DNA or mRNA by Watson-Crick pairing based on the known sequence of the EMAP II DNA as described in U.S. Patent No. 5,641,867 to Stern et al., the disclosure of which is incorporated by reference herein in its entirety. For example, an antisense oligonucleotide of the invention may consist of a 4, 6 or 8 or more nucleotide oligonucleotide having a base sequence corresponding to the EMAP II DNA sequence disclosed in Stern et al., *supra*, up to 20, 30, or 40 nucleotides in length, or even the full length of the DNA sequence. In addition, such compounds may be identified in accordance with known techniques as described below.

The inhibiting step may be carried out by administering an EMAP II receptor antagonist to the subject in an amount effective to stimulate vascular growth in the lungs of the subject. EMAP II receptor antagonists may be identified in accordance

with known techniques, but are in general analogs of EMAP II, such as EMAP II having three to five N-terminal and/or C-terminal amino acids deleted.

Active compounds useful for effecting the aforesaid inhibiting steps may be administered by any suitable means, including intraperitoneal, subcutaneous, intraarterial, intravenous, intramuscular, and intrathecal injection. Injection may be through a syringe, through a canula or catheter into a desired vessel or organ, etc.. The compounds may be administered by inhalation into the airways, and particularly the alveoli, of the lungs, such as by the inhalation of respirable aerosol particles (e.g., 1 to 5 micron diameter particles) comprising the active compound.

Pharmaceutical formulations of the invention typically comprise an active compound selected from the group consisting of compounds that specifically bind to EMAP II (e.g., an antibody as described above), compounds that inhibit the expression of EMAP II, and EMAP II receptor antagonists; and a pharmaceutically acceptable carrier. Any pharmaceutically acceptable carrier may be employed, such as sterile saline solution, sterile water, etc. The active compound is included in the pharmaceutically acceptable carrier in any suitable amount, such as between about .001, .005 or .01 percent by weight to about 10, 20 or 50 percent by weight.

Dosage of the active compound will depend upon the particular active compound, the route of administration, the particular disorder being treated, the age, weight, and condition of the subject, etc. For example, for antisense oligonucleotides, the dosage is preferably one which produces intracellular concentrations of the oligonucleotide of from 0.05 to 50  $\mu$ M. typically the dosage to a human will be from about 0.01, 0.1 or 1 mg/Kg up to 50, 100, or 150 mg/Kg. In an additional example, for antibodies, the dosage is typically 0.01, 0.05 or 0.1 up to 20, 40 or 60 mg/Kg.

Active compounds that are nucleotides or proteins (e.g., antibodies) may be administered either directly as described above or through a vector intermediate that expresses the same in the subject. Thus vectors used to carry out the present invention are, in general, RNA virus or DNA virus vectors, such as lentivirus vectors, papovavirus vectors (e.g., SV40 vectors and polyoma vectors), adenovirus vectors and adeno-associated virus vectors. See generally T. Friedmann, Science **244**, 1275 (June 1989). Examples of lentivirus vectors that may be used to carry out the present invention include Moloney Murine Leukemia Virus vectors, such as those described in U.S. Patent No. 5,707,865 to Kohn. Any adenovirus vector can be used to carry

out the present invention. See, e.g., U.S. Patent No. 5,518,913, U.S. Patent No. 5,670,488, U.S. Patent No. 5,589,377; U.S. Patent No. 5,616,326; U.S. Patent No. 5,436,146; and U.S. Patent No. 5,585,362. The adenovirus can be modified to alter or broaden the natural tropism thereof, as described in S. Woo, *Adenovirus redirected*, Nature Biotechnology 14, 1538 (Nov. 1996). Any adeno-associated virus vector (or AAV vector) can also be used to carry out the present invention. See, e.g., U.S. Patent No. 5,681,731; U.S. Patent No. 5,677,158; U.S. Patent No. 5,658,776; U.S. Patent No. 5,658,776; U.S. Patent No. 5,622,856; U.S. Patent No. 5,604,090; U.S. Patent No. 5,589,377; U.S. Patent No. 5,587,308; U.S. Patent No. 5,474,935; U.S. Patent No. 5,436,146; U.S. Patent No. 5,354,678; U.S. Patent No. 5,252,479; U.S. Patent No. 5,173,414; U.S. Patent No. 5,139,941; and U.S. Patent No. 4,797,368. The regulatory sequences, or the transcriptional and translational control sequences, in the vectors can be of any suitable source, so long as they effect expression of the heterologous nucleic acid in the target cells. For example, commonly used promoters are the LacZ promoter, and promoters derived from polyoma, Adenovirus 2, and Simian virus 40 (SV40). See, e.g., U.S. Patent No. 4,599,308. The heterologous nucleic acid may encode any product that inhibits the expression of the EMAP II gene in cells infected by the vector, such as an antisense oligonucleotide that specifically binds to the EMAP II mRNA to disrupt or inhibit translation thereof, a ribozyme that specifically binds to the EMAP II mRNA to disrupt or inhibit translation thereof, or a triplex nucleic acid that specifically binds to the EMAP II duplex DNA and disrupts or inhibits transcription thereof. All of these may be carried out in accordance with known techniques, as (for example) described in U.S. Patents Nos. 5,650,316; 5,176,996, or 5,650,316 for triplex compounds, in U.S. Patents Nos. 5,811,537; 5,801,154; and 5,734,039 for antisense compounds, and in U.S. Patents Nos. 5,817,635; 5,811,300; 5,773,260; 5,766,942; 5,747,335; and 5,646,020 for ribozymes (the disclosures of which are incorporated by reference herein in their entirety). The length of the heterologous nucleic acid is not critical so long as the intended function is achieved, but the heterologous nucleic acid is typically from 5, 8, 10 or 20 nucleic acids in length up to 20, 30, 40 or 50 nucleic acids in length, up to a length equal the full length of the EMAP II gene. Once prepared, the recombinant vector can be reproduced by (a) propagating the vector in a cell culture, the cell culture comprising cells that permit the growth and reproduction of the vector therein; and then (b) collecting the recombinant vector from the cell culture, all in accordance with known



techniques. The viral vectors collected from the culture may be separated from the culture medium in accordance with known techniques, and combined with a suitable pharmaceutical carrier for administration to a subject. Such pharmaceutical carriers include, but are not limited to, sterile pyrogen-free water or sterile pyrogen-free saline solution. If desired, the vectors may be packaged in liposomes for administration, in accordance with known techniques.

Any suitable route of administration can be used to carry out the present invention, depending upon the particular condition being treated. Suitable routes include, but are not limited to, intravenous, intrarterial, intrathecal, intraperitoneal, intramuscular, and intralesional injection. Intralesional injection is currently preferred.

The dosage of the recombinant vector administered will depend upon factors such as the particular disorder, the particular vector chosen, the formulation of the vector, the condition of the patient, the route of administration, etc., and can be optimized for specific situations. In general, the dosage is from about  $10^7$ ,  $10^8$ , or  $10^9$  to about  $10^{11}$ ,  $10^{12}$ , or  $10^{13}$  plaque forming units (pfu).

In addition to their pharmaceutical or veterinary use, the recombinant vectors of the present invention (sometimes also referred to as "active agents" herein) are useful *in vitro* to distinguish cells in culture based on their response to the active agents, to induce apoptosis, etc. Such techniques are useful for both carrying out cell culture procedures and for drug screening purposes.

*In vitro* methods of screening compounds for efficacy in carrying out the methods of treatment described above are also disclosed herein. In general, in one embodiment, such methods comprise determining *in vitro* whether the compound inhibits the expression of EMAP II (preferably the mammalian gene, and most preferably the human gene). The inhibition of expression of EMAP II indicates the compound is useful in the methods of treatment described above. Numerous such screening methods are available. The methods can be carried out in a cell or cells, or can be carried out in essentially cell free preparation. The method can be carried out by screening for compounds that specifically disrupt either transcription or translation of EMAP II. The compound to be screened may be a member of a library of compounds (the term "compound" as used in this respect referring to both small organic compounds and other therapeutic agents such as recombinant viral vectors).

The method may be carried out as a single assay, or may be implemented in the form of a high throughput screen in accordance with a variety of known techniques.

In another embodiment, the method of screening compounds comprises determining *in vitro* whether said compound specifically binds to EMAP II (including fragments thereof) (preferably the mammalian gene product; most preferably the human gene product). The determining step can be carried out by screening for binding of a test compound or probe molecule to the entire full length EMAP II gene product, or to a peptide fragment thereof (*e.g.*, a fragment of from 5, or 10 amino acids in length up to the full length of EMAP II). The binding of the compound to the EMAP II indicates that the compound is useful in the methods of treatment described herein. Such techniques can be carried out by contacting a probe compound to EMAP II or a fragment thereof in any of the variety of known combinatorial chemistry techniques (including but not limited to split pool techniques, chip-based techniques and pin-based techniques). Any suitable solid support can be used to immobilize the EMAP II or a fragment thereof to find specific binding partners thereto (or immobilize the members of the library against which the EMAP II or fragment thereof is contacted to find specific binding partners thereto), and numerous different solid supports are well known to those skilled in the art. Examples of suitable materials from which the solid support may be formed include cellulose, pore-glass, silica gel, polystyrene, particularly polystyrene cross-linked with divinylbenzene, grafted copolymers such as polyethyleneglycol/ polystyrene, polyacrylamide, latex, dimethylacrylamide, particularly cross-linked with N,N'-bis-acryloyl ethylene diamine and comprising N-t-butoxycarbonyl-beta-alanyl-N'-acryloyl hexamethylene diamine, composites such as glass coated with a hydrophobic polymer such as cross-linked polystyrene or a fluorinated ethylene polymer to which is grafted linear polystyrene, and the like. Thus the term "solid support" includes materials conventionally considered to be semi-solid supports. General reviews of useful solid supports that include a covalently-linked reactive functionality may be found in Atherton et al., *Prospectives in Peptide Chemistry*, Karger, 101-117 (1981); Amamath et al., *Chem. Rev.* 77: 183 (1977); and Fridkin, *The Peptides*, Vol. 2, Chapter 3, Academic Press, Inc., pp 333-363 (1979). The solid support may take any suitable form, such as a bead or microparticle, a tube, a plate, a microtiter plate well, a glass microscope cover slip, etc.

The present invention can be used with probe molecules, or libraries (where groups of different probe molecules are employed), of any type. In general, such probe molecules are organic compounds, including but not limited to that may be used to carry out the present include oligomers, non-oligomers, or combinations thereof.

5 Non-oligomers include a wide variety of organic molecules, such as heterocyclics, aromatics, alicyclics, aliphatics and combinations thereof, comprising steroids, antibiotics, enzyme inhibitors, ligands, hormones, drugs, alkaloids, opioids, benzodiazepenes, terpenes, porphyrins, toxins, catalysts, as well as combinations thereof. Oligomers include peptides (that is, oligopeptides) and proteins,  
10 oligonucleotides (the term oligonucleotide also referred to simply as "nucleotide, herein) such as DNA and RNA, oligosaccharides, polylipids, polyesters, polyamides, polyurethanes, polyureas, polyethers, poly (phosphorus derivatives) such as phosphates, phosphonates, phosphoramides, phosphonamides, phosphites, phosphinamides, etc., poly (sulfur derivatives) such as sulfones, sulfonates, sulfites,  
15 sulfonamides, sulfenamides, etc., where for the phosphorous and sulfur derivatives the indicated heteroatom for the most part will be bonded to C, H, N, O or S, and combinations thereof. Numerous methods of synthesizing or applying such probe molecules on solid supports (where the probe molecule may be either covalently or non-covalently bound to the solid support) are known, and such probe molecules can  
20 be made in accordance with procedures known to those skilled in the art. *See, e.g.*, U.S. Patent No. 5,565,324 to Still et al., U.S. Patent No. 5,284,514 to Ellman et al., U.S. Patent No. 5,445,934 to Fodor et al. (the disclosures of all United States patents cited herein are to be incorporated herein by reference in their entirety).

Test compounds used to carry out the present invention may be of any type,  
25 including both oligomers or non-oligomers of the types described above in connection with probe molecules above. Again, such test compounds are known and can be prepared in accordance with known techniques.

Where multiple different probe molecules are desired to be tested, a screening substrate useful for the high throughput screening of molecular interactions, such as in  
30 "chip-based" and "pin-based" combinatorial chemistry techniques, can be prepared in accordance with known techniques. All can be prepared in accordance with known techniques. *See, e.g.*, U.S. Patent No. 5,445,934 to Fodor et al., U.S. Patent No. 5,288,514 to Ellman, and U.S. Patent No. 5,624,711 to Sundberg et al.

In the alternative, screening of libraries of probe molecules may be carried out with mixtures of solid supports as used in "split-pool" combinatorial chemistry techniques. Such mixtures can be prepared in accordance with procedures known in the art, and tag components can be added to the discreet solid supports in accordance with procedures known in the art. *See, e.g.*, U.S. Patent No. 5,565,324 to Still et al.

The present invention is explained in greater detail in the following non-limiting examples.

## EXAMPLE 1

### EMAP II Inhibits Lung Neovascularization,

#### Epithelial Morphogenesis and Epithelial-Mesenchymal Interactions

Neovascularization is crucial to lung development and is mediated through a variety of angiogenic and anti-angiogenic factors. Herein, it is shown that excess Endothelial Monocyte Activating Polypeptide (EMAP) II, an anti-angiogenic protein, not only inhibits fetal lung neovascularization, but also significantly alters lung epithelial morphogenesis. In a murine xenograft model of lung neovascularization and morphogenesis, embryonic lungs transplanted under the skin of immunocompromised mice receiving intraperitoneal EMAP II, had a 56% reduction in vessel density ( $p < 0.0001$ ) compared to control. EMAP II treated lung transplants exhibited a marked alteration in lung morphogenesis, including lack of type II alveolar cell formation. In contrast, lung implants in animals receiving a blocking antibody to EMAP II had an increase in vessel density of 50% ( $p < 0.0001$ ) and most distal epithelial cells expressed surfactant protein C. Co-cultures of embryonic epithelial and mesenchymal cells showed that EMAP II expression is localized to the peri-epithelial cyst region. Exposure of these co-cultures to excess EMAP II inhibited epithelial cyst formation by 71% ( $p < 0.0001$ ); while, conversely, EMAP II antibody increased cyst formation by 54% ( $p < 0.0001$ ). There was a time-dependent induction of apoptosis by EMAP II limited to the epithelial cells in the co-culture system that was confirmed by apoptosis induction in the epithelial cells of the explant model. These studies demonstrate that EMAP II modulates vessel growth in the developing lung, inhibition of vessel growth, results in altered lung morphogenesis, and effects epithelial - mesenchymal interactions where, in the absence of vascular growth it induces apoptosis. Therefore, EMAP II, negatively modulates lung

neovascularization as well as leading to the arrest of lung epithelial morphogenesis and apoptosis.

## I. EXPERIMENTAL PROCEDURES.

5           **Synthesis of Recombinant (r) EMAP II from E. Coli and generation of a peptide antibody.** The cDNA of mature human EMAP II was cloned from RT-PCR products of U937 cells total RNA based on primers obtained from gene bank (accession #10119) into TA vector (Invitrogen). Confirmation of the clones was provided by sequence analysis, after which the cDNA was inserted into PET28a, 6 X  
10 his-tag containing plasmid. E. coli. (DE<sub>3</sub>) underwent transformation with the EMAP II/PET28a plasmid and were induced with 1-4 mM IPTG. After 3-4 hours of induction, the cells were pelleted, lysed and the EMAP II protein was purified through the use of a nickel column as per protocol (Qiagen) with all procedures performed at 4°C. Briefly, pelleted cells were lysed with 50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 300 mM NaCl,  
15 and 10 mM imidazole in the presence of lysozyme of 1 mg/ml. Following sonication, cellular debris are removed by centrifugation prior to being loaded on the Ni-NTA slurry. Following washing of the column, rEMAP II is eluted off with 8M urea, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, and 0.01 M Tris·Cl pH 5.9. Purified rEMAP II is dialyzed at 4°C against PBS three times prior to being aliquoted and frozen at -80°C. When an  
20 aliquot of rEMAP II was thawed, it was used immediately for experiments (it was not refrozen and used in future studies). This is essential to maintain rEMAP II's activity.

A peptide sequence of 13 amino acid residues located within a homologous region of the human and murine forms of mature EMAP II were used to generate an antibody. This peptide was synthesized and the antibody produced by Zymed  
25 Laboratories Inc. as per protocol and is used for immunohistochemistry and western blotting. The antibody is specific to EMAP II identified by producing a single band on a western blot that is blocked after being incubated with excess EMAP II (data not shown).

**Isolation of epithelial and mesenchymal cells for co-culture.** Organotypic  
30 murine lung cultures were performed following the protocol of Schuger et al. [Schuger, *Development* **110**, 1091-9 (1990); Schuger, *J. Cell. biol.* **139**, 553-62 (1997); Schuger, *Int. J. Dev.Biol.* **42**, 217-220 (1998)]. In brief, timed gestation 15d embryos underwent dissection from Swiss-Webster mice (Simonsen, Morgan Hill, CA), lungs were isolated, underwent digestion in PBS containing 0.3% trypsin and

0.1% EDTA for 10 minutes at 37°C prior to being filtered through a 100 µm-pore mesh. The mixed epithelial-mesenchymal cells were then resuspended in minimal essential medium (MEM:Gibco-BRL) with nonessential amino acids and plated at a concentration of  $2-2.5 \times 10^6$  cells / ml in 8 well chamber slides. Experiments were performed in the presence of vehicle, rEMAP II (mature 0.8-3.2 µg/ml), EMAP II peptide antibody (3-6 µg/ml), and rabbit IgG (control). Epithelial cyst formation was evaluated by counting the number of epithelial cyst per high power field (HPF), we analyzed 10 fields per condition and averaged them.

**Xenograft lung transplant model.** Timed pregnant Swiss Webster mice at gestational day 12 (based on appearance of vaginal plug = day 0) were obtained, housed, and handled according to a protocol approved by the animal care committee at CHLARI (Childrens Hospital of Los Angeles Research Institute). On day 14.5 dams were sacrificed and the embryo removed. The lungs and heart were withdrawn as a block microdissection and placed in ice cold PBS. The heart was then removed and the lung was placed on top of a 0.80 µm Millipore filter disk (Millipore) and implanted into a dorsal skinfold chamber of a nude mouse using sterile technique. The skin was closed with skin staples. A sibling lung was used for histological analysis and comparison to the implanted lung. Nude mice were then injected intraperitoneal by (IP) on a daily basis with either vehicle (phosphate buffered saline - PBS and albumin), EMAP II (1µg/day), rabbit IgG or EMAP II antibody (25 or 50 µg / every three days).

**RT-PCR of lung transplants.** Following 14 days, lung xenografts were removed from mice that had been treated with rEMAP II, antibody to EMAP II, or vehicle, separated from the carrier mouse skin, total RNA was extracted by RNA STAT-60 (Tel-Test "B", Inc., Friendswood, TX) and the RNA of the transplanted lungs were reverse transcribed by superscript II RNase H - reverse transcriptase (GIBCO-BRL) using 3 mcg of total RNA template, 4 µl of 5 X RT buffer, 2 µl of 0.1 M DTT, 0.5 µg of target gene specific 3' primer in a total reaction volume of 18 µl. The reaction mix was incubated at 70°C for 10 minutes followed by incubation on ice 2 minutes. One µl of 10 mM dNTP, 1µl of superscript II RNase H - reverse transcriptase were added. The mixture was incubated at 49°C for 1 hour and 30 minutes followed by 70°C for 10 minutes. The first strand cDNAs thus synthesized were used directly for PCR amplification of the target cDNA. The target cDNA primers were: 1) murine PECAM-1 5' primer - 5' GTC ATG GCC ATG GTC GAG

TA 3' (SEQ ID NO: 1) and the 3' primer - 5' CTC CTC GGC ATC TTG CTG AA 3' (SEQ ID NO: 2), 2) murine tie-2 5' primer - 5' TTG AAG TGA CGA ATG AGA T 3' (SEQ ID NO: 3) and the 3' primer - 5' ATT TAG AGC TGT CTG GCT T 3' (SEQ ID NO: 4), 3) murine SP-C 5' primer - 5'-CAT ACT GAG ATG GTC CTT GAG-3' (SEQ ID NO: 5), and 3' primer - 5'-TCT GGA GCC ATC TTC ATG ATG-3' (SEQ ID NO: 6) and 4) murine T1- $\alpha$  5' primer - 5' GAA CAT GAG AGT ACG ACC ACT GTC AAA 3' (SEQ ID NO: 7) and the 3' primer - 5' TTA GGG CGA GAA CCT TCC AGA AAT CTT 3' (SEQ ID NO: 8).  $\beta$  Actin, used as the house keeping gene, was performed on all the samples using the primers: 5' primer - 5' GTA TGG AAT CCT GTG GCA TCC 3' (SEQ ID NO: 9) and the 3' primer - 5' TAC GCA GCT CAG TAA CAG TCC 3' (SEQ ID NO: 10). In addition, controls were performed on all targeted cDNA sequences using primer pairs without the presence of the first - strand cDNA template. Target cDNA segments were amplified using 1/10th of the above first - strand cDNA template, 10  $\mu$ l of 10 X buffer, 0.5  $\mu$ l of 10 mM dNTP's, 300 ng of each of 5' and 3' end specific primers, and 1 unit of Taq Polymerase (Stratagene) in a 50  $\mu$ l reaction. The PCR program was 94°C 1 min., 62°C 30 sec., and 72°C 30 sec. for 30 cycles. Equal amounts of all amplification cDNA fragments were analyzed by agarose gel electrophoresis, photographed, and analyzed.

**In situ hybridization and construction of cDNA probes.** Total RNA was extracted from 15 day gestation mouse lung tissue by RNA STAT-60 (Tel-Test "B", Inc., Friendswood, TX). RNA (3  $\mu$ g) was incubated with oligo(dT) primer for 10 minutes at 70°C. First-strand cDNA synthesis was performed according to manufacturer's instructions (GIBCO BRL, Grand Island, NY). After first-strand synthesis, cDNA was generated by PCR amplification with 10 pmol of specific primers for 30 cycles of amplification (94°C 1', 62°C 1', 72°C 1'). The primers used were as follows: SP-C, sense, 5'-CAT ACT GAG ATG GTC CTT GAG-3' (SEQ ID NO: 11), and antisense, 5'-TCT GGA GCC ATC TTC ATG ATG-3' (SEQ ID NO: 12). The RNA probe for EMAP II was 456 bp in size and obtained from a region that has minimal homology with other known proteins.. The generated SP-C PCR product was subcloned into TA vector (Invitrogen, Carlsbad, CA) for the in vitro transcription of RNA.

**Digoxigenin RNA probe labeling by in vitro transcription.** DNA of the SP-C subclone, in good orientation for in vitro transcription of antisense RNA by T7 RNA polymerase, was linearized by Hind III digestion and used as a template for probe labeling. Antisense RNA probe labeling with digoxigenin-UTP by in vitro transcription with T7 RNA polymerase was performed as per manufactures instructions (DIG RNA labeling kit, Boehringer Mannheim, Indianapolis, IN).

**RNA in situ hybridization (RISH) using DIG-labeled cRNA probes.** Murine embryo control lung day 14 g.a. and murine transplants, days 14 g.a. + 3.5, 14 g.a. + 7, 14 g.a. + 10.5, and 14 g.a. + 14 were obtained for in situ hybridization. The Dig RNA probe anti-sense and sense (control) were made using the Dig RNA labeling Kit (SP6 / T7) from Boehringer Mannheim (Indianapolis, IN). RISH was performed on 5-mm paraffin embedded material sections according to nonradioactive in situ hybridization application manual (Boehringer Mannheim, Indianapolis, IN). Using DEPC treated equipment and solutions, paraffin embedded specimens underwent sectioning, rehydration and incubation in a prewarmed 5µg/ml proteinase K solution. Slides were then reimmersed in 4% PFA, treated with a 0.25% acetic anhydride and dehydrated. Sections were exposed to a hybridization solution containing 50% formamide, 10% dextran sulfate, 1mg/ml tRNA, 1 X Denhardt's solution, 4 X SSC, 50mM Tris and 5mM EDTA that contained 150-300ng/ml of dig-labeled RNA probe at 50°C overnight. Slides were washed at 55°C in 2 X SSC / 50% formamide, 1 X SSC and 0.1 SSC for 30 minutes prior to being incubated with RNase A (20µg/ml) for 30 minutes at 37°C. After being rinsed with 2 X SSC and Dig Nucleic Acid detection was accomplished using the Genius 3 kit from Boehringer Mannheim. Briefly, slides were incubated in 0.1M Maleic acid / 0.15M NaCl pH 7.5 for 5 minutes after which they underwent blocking in a 1% block reagent. Following blocking, slides were incubated with anti-Dig-AP conjugate at 4°C overnight, rinsed, and incubated with a dilute NBT / BCIP solution for 3 hours a room temperature. Slides then underwent counterstaining with a 0.02% fast green solution for 2 minutes, rinsed in water, air dried and mounted. Hybridization with sense probe or without probe was performed as negative control and they always showed no signals. All sections were examined and photographed under light microscopy.

**Histologic and immunohistochemistry analysis of the murine lung transplants.** Following 14 days, lung xenografts were removed from mice that had



been treated with rEMAP II, blocking antibody to EMAP II, or vehicle, separated from the carrier mouse skin, fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded (during all procedures, DEPC water and precaution against RNases were taken). Fixed tissue was sectioned at 5 micron intervals. The lung transplants then  
5 underwent H & E staining for structural analysis. For immunolocalization of PECAM-1 antigens (Pharmigen, San Diego, CA), a rat anti-murine PECAM-1 antibody (4 µg/ml) was employed. Tissues were deparaffinized and underwent peroxide quenching. Using a histostain kit from Zymed (San Francisco, CA), after blocking, the sections were exposed to the primary antibody overnight at 4°C.  
10 Sections were then incubated with secondary biotinylated antibody as per the manufacturer's protocol. A brief incubation with the Streptavidin-HRP conjugate system (Zymed) was followed by development using the chromogen substrate aminoethylcarbazole. Periodic Acid Schiff (PAS) stain was performed, using a kit from Sigma (St. Louis, MO) according to the manufactures instructions.

15 **TUNEL analysis of fetal epithelial - mesenchymal cell co-cultures.** The spatial induction of apoptosis was analyzed in epithelial - mesenchymal cell co-culture or lung xenografts using the In Situ Death Detection Kit from Boehringer Mannheim. In brief, co-cultured cells were exposed to vehicle, EMAP II (3.2 µg/ml), EMAP II antibody (6 µg/ml) or rabbit IgG. Cells were evaluated on days one to three  
20 for apoptosis. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X and exposed to the TUNEL reaction (containing terminal deoxynucleotidyl transferase and a nucleotide mixture in a reaction buffer). After which, the cells were exposed to a fluorescein antibody, counterstained with propidium iodine (0.05 µg/ml), mounted with PBS / glycerol, and observed under a floursecent microscope  
25 (Olympus). Lung xenografts were fixed in 4% paraformaldehyde, dehydrated and paraffin embedded. 5 micron sections were cut, rehydrated and prior to exposure to the TUNEL reaction. Apoptotic cells were revealed using alkaline phosphatase and observed under light microscopy.

30 **Statistics:** Statistical analysis was performed using student t-test on the computer program Statview.

## II. RESULTS.

**Purification of recombinant EMAP II.** In order to determine the function of EMAP II in the developing lung, it was important to develop an easy and reproducible production system for recombinant EMAP II. We used a PET28a 6X His-tag system to quickly and efficiently isolate mature rEMAP II under native conditions. Recombinant (r) EMAP II was expressed in *E. coli* (shown in the Coomassie blue gel, 1st column **Fig. 1**), induced with 1-4 mM IPTG and the *E. coli* pelleted after 3-4 hours of induction (2nd column **Fig. 1**). The purified, recombinant mature form of EMAP II (column 3, **Fig. 1**) had MR 23kDa (with the 6X His-tag) on both reduced and nonreduced SDS-PAGE. Activity of rEMAP II, measured by induction of TNF- $\alpha$  and monocyte migration [Kao, 1994 #44], was found to be closely analogous to that previously observed with meth A-derived EMAP II. LPS levels were < 15 pg/ml as measured with a LAL kit (Biowhittaker QCL-1000). Heat-treated EMAP II was inactive in these assays. The peptide antibody generated in a rabbit, is specific to EMAP II, identified by producing a single band on Western analysis that is blocked after being incubated with excess EMAP II (data not shown).

**EMAP II inhibition of fetal lung vascular development.** To better define EMAP II's role in embryonic lung neovascularization, murine lungs obtained at gestational age 14.5 days, were implanted subcutaneously into nude mice. Mice then received either vehicle or rEMAP II (1 $\mu$ g/day) IP every day for 14 days. A separate group of mice were treated with either EMAP II blocking antibody (25 or 50 $\mu$ g) or rabbit IgG every 3 days for 14 days. Lung transplants were then excised and evaluated for vascular and structural development using PECAM-1 and hematoxylin and eosin staining respectively. Compared to lung xenografts implanted in mice treated with vehicle alone, implants in mice receiving the anti-angiogenic protein EMAP II exhibited a striking 56% reduction in lung vessel formation. Differences between lung vessel formation (assessed by counting the number of vessels identified per high powered field (HPF) with PECAM-1 antibody) in control (**Fig. 2A**) and EMAP II treated (**Fig. 2B, D**) animals were highly statistically significant by student t-test ( $p < 0.0001$ ). In contrast, animals receiving blocking antibody to EMAP II had a significant dose dependent increase of 50% ( $p < 0.0001$ ) in vessel counts per HPF (**Fig. 2C, E**) ( $n=10$  / group, performed on 3 separate occasions). Consistent with these histologic findings, mRNA harvested from lung xenografts of animals treated

with rEMAP II demonstrated a reduction in PECAM-1 and Tie-2 by RT-PCR compared to control. Converse results, an increase in PECAM-1 and Tie-2 PCR products, were obtained from xenografts in animals treated with the blocking EMAP II antibody (Fig. 2F). Negative controls for PCR amplification of the PECAM-1 and Tie-2 transcripts, without RT, demonstrated no specific PCR product in each rxn (data not shown).

**EMAP II inhibits epithelial maturation.** It was postulated that pulmonary vascularization might influence epithelial cell differentiation. After administration of rEMAP II, histologic analysis of lung xenografts in these mice showed a marked inhibition of structural maturation (Fig. 3D-F) compared to vehicle treated animals (Fig. 3A-C). This was demonstrated by a lack of well-defined bronchi with characteristic epithelium (Fig. 3A), or of distal airways with attenuated epithelium consistent with alveolar epithelium, as compared to those xenografts where the mouse received vehicle alone (Fig. 3B,C). In addition, lung xenografts in mice treated with EMAP II had alveolar epithelial cells that appeared dysplastic (Fig. 3E,F) and an apparent stasis in respiratory duct formation (Fig. 3D, arrows) as compared to those transplants in mice receiving vehicle alone (3A). To discern whether morphologic progression actually occurred, we assessed the xenografts for markers of distal lung morphogenesis. Lung xenografts in mice receiving vehicle alone underwent type II alveolar cell differentiation as marked by SP-C expression (Fig. 4A,B). In contrast, lung xenografts in animals receiving EMAP II had a marked reduction in SP-C expression throughout the entire transplanted lung, even in the most peripheral airways (Fig. 4C,D). Further supporting our findings, animals receiving blocking EMAP II antibody had a strikingly increased number of type II cells, with essentially every distal epithelial cell expressing SP-C (Fig. 4E,F). Therefore, it appeared that excess EMAP II lead to profound inhibition of peripheral lung epithelial morphogenesis and differentiation.

Consistent with the *in situ* hybridization findings, mRNA harvested from lung xenografts of animals treated with rEMAP II demonstrated a reduction in SP-C by RT-PCR compared to controls. In contrast, animals treated with the blocking EMAP II antibody exhibited an increase in the SP-C amplicon confirming the *in situ* results (Fig. 4G). Interestingly, T1- $\alpha$  a type I alveolar epithelial cell marker, was slightly elevated in xenografts treated with rEMAP II, whereas a marked reduction in T1- $\alpha$

was found in the blocking EMAP II antibody treated lungs, the inverse of the high level of SP-C expression, a type II cell marker (Fig. 4G). Negative controls for PCR amplification of the SP-C and T1- $\alpha$  transcripts, without RT, demonstrated no specific PCR product in each rxn (data not shown).

5 We also evaluated glycogen production in the lung xenografts. Xenografts obtained from mice treated with rEMAP II demonstrated excess glycogen production (denoted by the magenta color) (Fig. 5D-F) compared to vehicle alone (Fig. 5A-C), further supporting the concept that EMAP II inhibited epithelial differentiation.

10 **EMAP II disruption of the epithelial-mesenchymal interface.** To further examine the role of the anti-angiogenic protein EMAP II in lung morphogenesis, the localization of EMAP II in epithelial - mesenchymal co-cultures was defined. Evaluation of lung epithelial-mesenchymal co-cultures after 3 days of incubation revealed EMAP II expression to be predominately in the peri-epithelial cyst region by both in situ hybridization (Fig. 6A) as well as immunohistochemistry (Fig. 6B)  
15 consistent with those results seen in fetal lung tissue [Schwarz, *Am. J. Physiol.* 276, L365-75 (1999)]. Interestingly, while EMAP II is expressed in epithelial and mesenchymal cells, its strongest expression is noted to be at the epithelial - mesenchymal junction as noted by the arrows in Figure 6A.

To determine the effect of EMAP II on epithelial cyst formation, epithelial-  
20 mesenchymal co-cultures were exposed to increasing concentrations of rEMAP II, EMAP II blocking antibody, or vehicle (PBS or rabbit IgG respectively). Epithelial cyst formation was analyzed as the total number of cyst formed per high power field (HPF). There was a dose-dependent, 71% inhibition ( $p < 0.0001$ ) of epithelial cyst formation and an alteration in structure in co-cultures exposed to EMAP II (Fig. 7B,  
25 D) as compared to control (Fig. 7A, arrows indicate normal epithelial cyst formation with the epithelial cells being surrounded by flattened laminin positive cells). Conversely, in the presence of the EMAP II blocking antibody (Fig. 7C, E) there was a 54% increase ( $p < 0.01$ ) in cyst formation that was also dose-dependent. Because we recently observed that EMAP II induces apoptosis in growing and dividing  
30 endothelial cells [Schwarz, *Journal. of Experimental. Medicine* 290 (1999)], we employed the TUNEL assay to determine whether induction of apoptosis due by EMAP II was responsible for the decrease in numbers of epithelial cyst. We found a time-dependent induction of apoptosis, starting in the peri-epithelial cyst region and

